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14. ABSTRACT

Mesenchymal stem cells (MSCs) differentiated from Induced pluripotent stem cells (iPSCs) have a potential application in clinic to treat osteoporosis and other skeletal diseases. Further engineering MSCs with homing factors like CXCR4 and osteogenic factors like shNoggin or FGF2 may increase the therapeutic effects. Toward these goals, we have generated iPSCs using lentiviral vectors from blood cells and integration-free iPSCs using episomal vectors. The generated iPSCs are pluripotent, as evident by expression of pluripotency markers and formation of teratoma. After differentiation of iPSCs into MSCs, the cells express MSC markers and can form bone nodule in in vitro culture. We will further test in year 2 whether iPSC-Derived MSCs that are genetically engineered with CXCR4 and other factors can increase systemic bone formation after transplantation in mice. We also proposed an alternative strategy for generating MSCs: direct conversion of blood cells into MSCs rather than reprogramming blood cells into iPSCs followed by re-differentiation.

15. SUBJECT TERMS
Induced pluripotent stem cells or iPS cells, mesenchymal stem cells

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INTRODUCTION

Developing strategies to treat osteoporosis and related bone disease is of significant relevance to the health care needs of the Armed Forces. The commonly used maintenance therapy has serious adverse effects after long-term use. MSC-based therapy is considered most promising for systemic augmentation of bone strength [1]. However, this approach has several limitations: 1) The available number of MSCs from one harvest is limited; 2) the osteogenic potential of MSCs from adult and aging patients is substantially lower than fetal MSCs [2]; 3) transplantation of stem cells that are engineered using retrovirus has a serious safety concern of insertional mutagenesis-associated tumorigenesis. A recent breakthrough of generating iPSCs from patient's own cells will provide a solution to all these potential problems [3]. iPSCs, unlike MSCs, can be expanded ex vivo to unlimited cell number, enabling us to use the prescreened best clone for therapy. Of note, generation of iPSC can rejuvenate the source cells from which iPSCs are derived, as evidenced by increased telomere [4]. Thus, we hypothesized that iPSCs-derived MSCs resemble more similarity to fetal MSCs in multi-potential differentiation abilities.

In year one, we have successfully generated mouse iPSCs using lentivirus and developed a novel episomal vectors for generating integration-free iPSCs, which are more clinically relevant and potentially safer than lenti-iPSCs. We also developed an approach for differentiation of iPSCs into functional MSCs that are capable of trilineage in vitro differentiation. We found that iPSC-MSCs can be easily transduced with lentivirus that expresses CXCR4 or shNoggin. These findings lay a foundation for our in vivo transplantation studies in year two.

BODY

1. Generation of mouse iPS cells using lentiviral vectors.

iPSCs are a type of pluripotent stem cell derived from non-pluripotent cells, typically adult somatic cells, by inducing forced expression of specific genes. In 2006, Yamanaka's group first converted fibroblasts into iPS cells by overexpressing 4 transcription factors—Oct4, Sox2, Myc and Klf4 [5]. These factors were subsequently used in the successful generation of hPSCs in 2007 [6]. This technical breakthrough has significant implications for regenerative medicine. The iPS cell technology lifts the big hurdle (i.e. immune rejection) in cell replacement therapy by providing MHC-identical autologous cells, which can be differentiated from patient-specific hPSCs. In addition, the "safe" clone that is engineered with therapeutic genes can be differentiated into HSCs for stem cell gene therapy.

To generate mouse iPSCs, we purified Sca1^{+} cells from bone marrow and transduced them with two lentiviral vectors that express OCT4 and SOX2, MYC and KLF4 (**Figure 1a**). Three days after transduction, cells were harvested for iPSC generation culture on the inactivated murine embryonic fibroblast feeder in the iPSC culture condition. Two weeks later, we observed the appearance of some mouse iPSC colonies (**Figure 1c**). We picked several colonies for further culture.

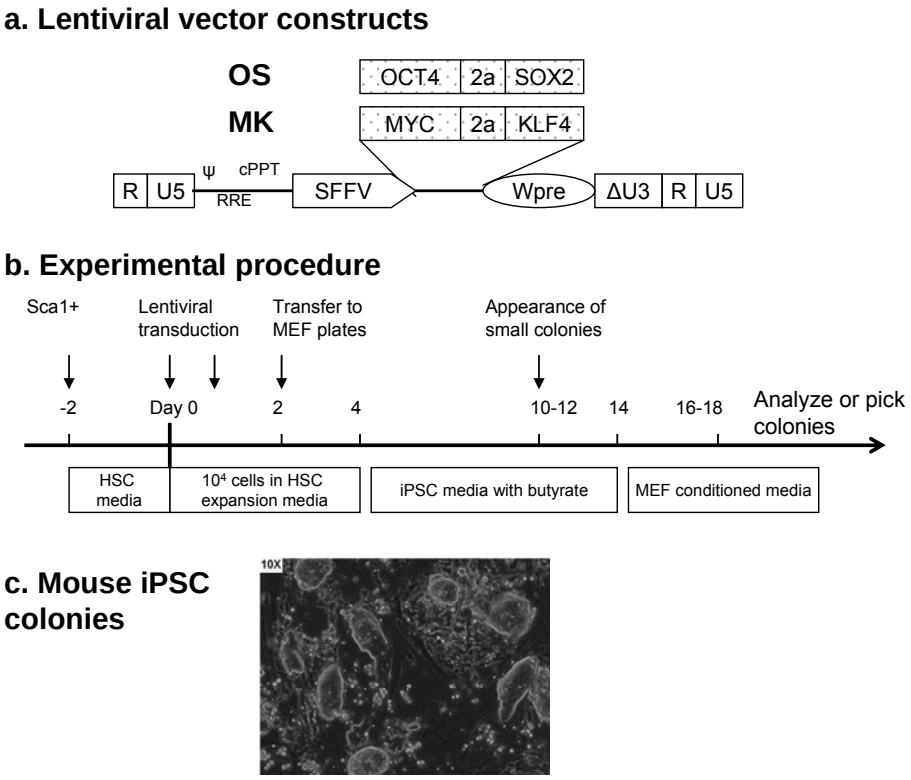
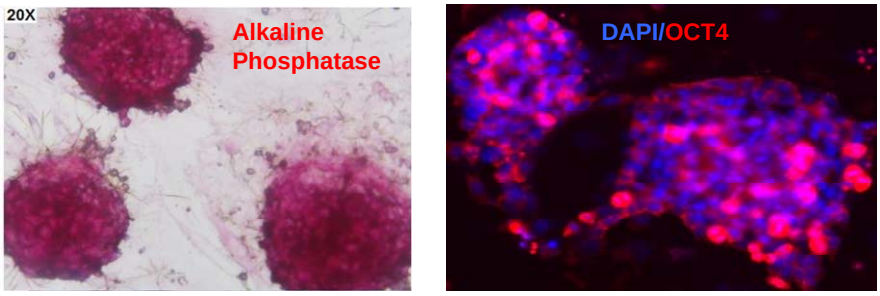


Figure 1. Generation of mouse induced pluripotent stem cells (iPSCs). A. Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of the reprogramming factors OCT4, SOX2, MYC andKLF4. Δ indicates the SIN design with partially deleted U3 of the 3′ long terminal repeat. cPPT, central polypurine tract; Wpre, posttranscriptional regulatory element; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; ψ, packaging signal; 2a, a self-cleavage site derived from equine rhinitis A virus. **B**. Experimental strategy for reprogramming mouse Sca1^{+} cells using lentiviral vectors. **C**. Shown are iPSC colonies.

2. Characterization of mouse iPS cells

To test whether the generated iPS cells are bona fide pluripotent stem cells, we performed immunostaining and teratoma assay. As shown in **Figure 2**, the iPS cell colonies expressed pluripotency markers like alkaline phosphatase, Oct4, Sox2, and Nanog. The formation of teratoma in mice is the gold standard of pluripotency of pluripotent stem cells. We subcutaneously injected 1x10^{6} iPS cells into each mouse. Four weeks after injection, teratomas were developed in all the mice (**Figure 2**). These data suggest that the generated iPS cells are pluripotent.



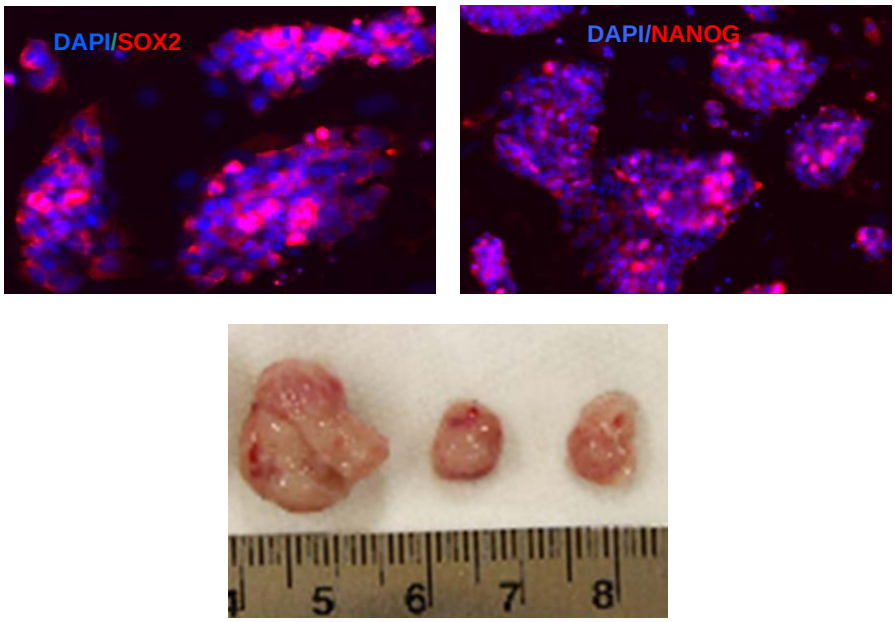


Figure 2. Characterization of mouse iPS cells. Immunostaining of iPS cells with stem cell markers alkaline phosphatase, Oct4, Sox2, and Nanog. **Bottom**: Teratomas from mice that were injected with iPS cells.

3 Generation of integration-free mouse iPS cells using episomal vectors.

For clinical applications, integration-free or footprint-free iPSCs need to be used to prevent potential adverse effects due to retroviral or lentiviral integration or due to the interference of residual expression of reprogramming factors on the differentiation of iPSCs into progenies of clinical interest. [7-8] Toward this goal, several approaches have been used for obtaining integration or transgene-free iPSCs, including the use of plasmids,[9] the Cre/loxP system, [10-11] adenoviruses, [12-13] piggyBac transposon, [14-15] minicircle DNA, [16] protein transduction, [17-18] sendai virus, [19] and miRNA. [20] However, these methods suffer from low efficiency, require repetitive induction or selection, or require virus production. Synthetic modified mRNA might solve the problem, [21] but it requires the daily addition of mRNA by lipofection.

Several investigators have used the EBNA1-based episomal vector due to its unique features: 1) only one transfection of vector DNA by nucleofection is needed for efficient reprogramming, and 2) the vector is lost in 5% or more cells after each cell division, leading to depletion of the episomal vector from cells after long-term passage. Recently, several groups have successfully used the pCEP4 episomal vector to generate footprint-free iPSCs. [22-24] However, in those studies, five to seven factors, including strong oncogenes like MYC and/or simian virus 40 large T antigen (SV40LT) were used, which raises safety concerns for clinical use of iPSCs.

We improved the vector design by using a strong promoter SFFV and including wpre in the vector, which substantially increased expression of reprogramming factors (**Figure 3**). After several tests, we have been successfully generated mouse iPSCs using episomal vectors. We will further culture and test these cells.

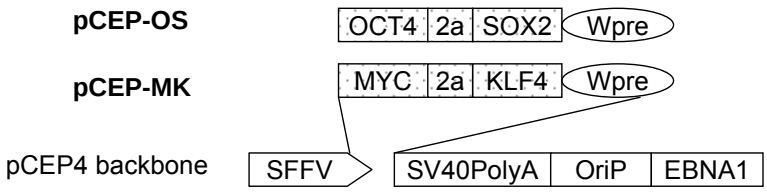


Figure 3. Schematic of episomal vectors used for generating integration-free iPSCs. Reprogramming factors were cloned into the pCEP4 backbone; their expression is driven by SFFV (Spleen focus-forming virus U3 promoter). 2a is a self-cleavage site derived from equine rhinitis A virus. Wpre, posttranscriptional regulatory element; SV40PolyA, polyadenylation signal from SV40 virus; OriP, EBV origin of replication; EBNA1, Epstein-Barr nuclear antigen 1, which plays essential roles in replication and persistence of episomal plasmid in infected cells.

4. Differentiation of mouse iPSCs into MSCs

After several tests, we have established a protocol that can efficiently differentiate mouse iPSCs into MSCs. iPSCs were harvested from MEF feeders and cultured in fibronectin-treated non-TC well plates with MEM10% FBS. Five days later,

the formed embryoid body (EB) were dissociated into single cells or small clumps with collagenase and cultured in gelatin-treated well plates in MesenCult® MSC Basal Medium (Stemcell Technologies). After several passages, we analyzed iPSC-MSCs by immunohistochemical staining. **Figure 4** shows that iPSC-derived MSCs express markers of mouse MSCs.

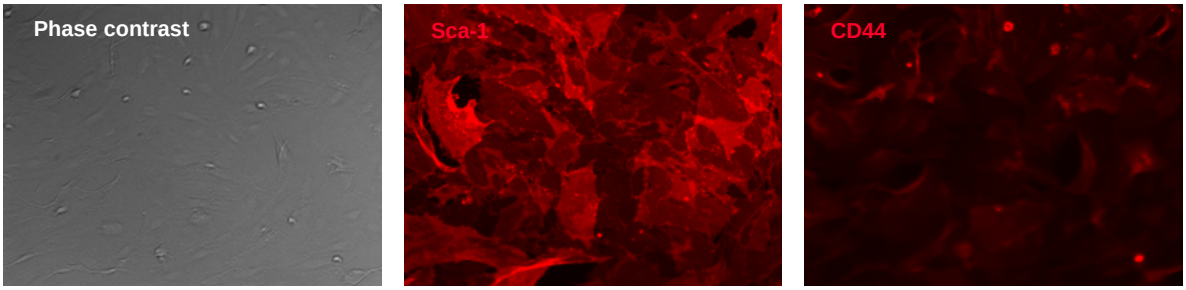


Figure 4. iPSC-derived MSCs manifest typical mesenchymal stem cell morphology and show high-level expression of typical mouse MSC markers Sca-1 and CD44.

We also tested the in vitro functionality of iPSC-derived MSCs using standard method. We found that these cells can be differentiated into adipocytes, osteogenic cells, and chondrocytes (**Figure 5**). These data suggest that our iPSC-MSCs are functional

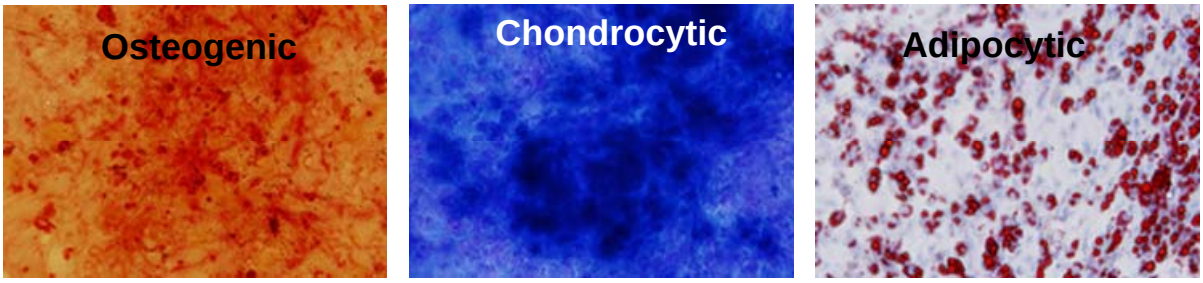


Figure 5. Multilineage differentiation capacity of iPSC-derived MSCs. iPSC-derived MSCs were culture in differentiation media for 3 weeks and then stained with Alizarin Red S staining for osteogenic differentiation, Alcian Blue staining for chondrocytic differentiation, and Oil Red O staining for adipocytic differentiation.

5. Derivation of mouse BM-MSCs

BM MSCs were isolated and cultured using standard protocols. Bone marrow cells from C57BL/6 mice were collected by flushing the femurs and tibias from 6–8-week-old mice with MEM medium supplemented with 5% heat-inactivated fetal calf serum (FCS) (Invitrogen, CA, USA). Erythrocytes-depleted bone marrow cells were plated in MEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Culture medium was changed at day 2 to remove nonadherent cells. Whole medium was subsequently replaced weekly. The cells were grown for 2–3 weeks until almost confluent. Adherent cells were then detached by Accutase treatment and replated using a 1:4 dilution. We can culture these BM MSCs for up to 2 months.

6. Transduction of MSCs with lentivirus

In preparation for in vivo transplantation, we cloned several lentiviral vectors for animal studies (**Figure 6**). In all the vectors, GFP is also expressed to facilitate examination of transplanted MSCs by flow cytometry. CXCR4 is a commonly used factor to promote stem cell homing to the marrow niche. shNoggin may promote osteoblastic differentiation. Recently, we found that systemic FGF2 can strongly increase bone formation [25].

We have generated lentivirus for these constructs. Co-culture of lentivirus with BM MSCs or iPSC-MSCs at an MOI of 1 led to a high transduction efficiency of ~95%.

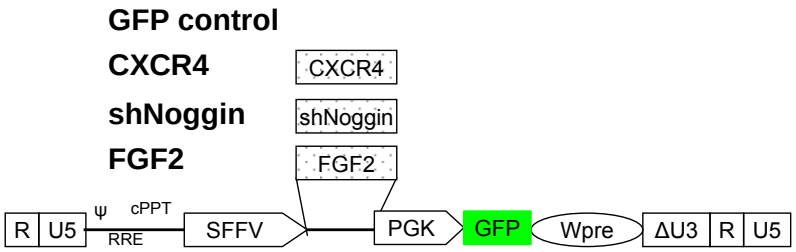


Figure 6. Lentiviral vectors for in vivo studies. Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of homing factor CXCR4 and osteogenic factors shNoggin and FGF2. Δ indicates the SIN design with partially deleted U3 of the 3′ long terminal repeat. cPPT, central polypurine tract; Wpre, posttranscriptional regulatory element; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; ψ, packaging signal. GFP expression is driven by PGK promoter.

7. Recommended changes

During our last year's culture of iPSC-MSCs, we occasionally observed undifferentiated iPSCs in the culture even after 10 passages of culture. This observation, together with similar reports in publications, calls upon a caution for the use of lentivirus generated iPSCs for therapy. As such, we have started to generate integration-free iPSCs for MSC differentiation. We hope this strategy will partially solve the concern of teratoma formation in clinical trials.

After submission of the grant application in 2010, exciting new findings have been published in the stem cell field. One novel approach to preventing residual undifferentiated iPSC-mediated teratoma formation has been proposed: direct conversion of somatic cells like fibroblasts or blood cells into cells of clinical interest such as liver cells, neural cells and cardimyeoctyes.[26-31] Therefore, we believe that direct transdifferentiation of peripheral blood (PB) cells into MSCs is scientifically more important than our original proposal because this will sidestep the generation of iPSCs. Once approved, we will conduct some preliminary test on transdifferentiation of human PB cells into MSCs.

KEY RESEARCH ACCOMPLISHMENTS

- Generated mouse iPSCs from hematopoietic cells with lentiviral vectors.
- Developed a novel episomal vector and generated integration-free iPSCs.
- Differentiated mouse iPSCs into functional MSCs.
- Cloned lentiviral vectors for in vivo study of iPSC-MSCs

REPORTABLE OUTCOMES

- Developed a novel episomal vector for generating integration-free iPSCs
- Developed a mouse integration-free iPSC cell line.
- The vectors developed in this study contributed to the publication of a paper in *Molecular Therapy*.
- This grant supported a technician.

CONCLUSION

We have generated mouse iPSCs from hematopoietic cells and differentiated iPSCs into functional MSCs. We also developed a novel episomal vector, which will have important applications in generating integration-free human iPSCs for clinical therapy. New findings in the past 2 years may have made our original proposal obsolete. We believe it is important to start to pursuit a better alternative strategy: directly converting blood cells into MSCs without iPSC generation.

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APPENDICES

The reprogramming vectors developed in this project have also been used for generating human iPSCs, which have been published in *Molecular Therapy* recently.

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Efficient Reprogramming of Human Cord Blood CD34+ Cells Into Induced Pluripotent Stem Cells With OCT4 and SOX2 Alone

Xianmei Meng[1], Amanda Neises[1], Rui-Jun Su[2,3], Kimberly J Payne[2,3], Linda Ritter[4], Daila S Gridley[4], Jun Wang[5], Matilda Sheng[1], K-H William Lau[6], David J Baylink[1] and Xiao-Bing Zhang[1]

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The reprogramming of cord blood (CB) cells into induced pluripotent stem cells (iPSCs) has potential applications in regenerative medicine by converting CB banks into iPSC banks for allogeneic cell replacement therapy. Therefore, further investigation into novel approaches for efficient reprogramming is necessary. Here, we show that the lentiviral expression of *OCT4* together with *SOX2* (OS) driven by a strong spleen focus-forming virus (SFFV) promoter in a single vector can convert 2% of CB CD34+ cells into iPSCs without additional reprogramming factors. Reprogramming efficiency was found to be critically dependent upon expression levels of OS. To generate transgene-free iPSCs, we developed an improved episomal vector with a woodchuck post-transcriptional regulatory element (Wpre) that increases transgene expression by 50%. With this vector, we successfully generated transgene-free iPSCs using OS alone. In conclusion, high-level expression of OS alone is sufficient for efficient reprogramming of CB CD34+ cells into iPSCs. This report is the first to describe the generation of transgene-free iPSCs with the use of OCT4 and SOX2 alone. These findings have important implications for the clinical applications of iPSCs.

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INTRODUCTION

The ability to generate induced pluripotent stem cells (iPSCs) from somatic cells has opened up a new avenue for regenerative medicine. Earlier studies used fibroblasts, such as those derived from a skin biopsy, to generate iPSCs by overexpression of Yamanaka factors (*OCT4, SOX2, MYC* and *KLF4*, or OSMK) or Thomson/Yu factors (*OCT4, SOX2, NANOG,* and *LIN28*).[1,2] However, it takes several weeks to prepare cells from a skin biopsy for reprogramming.[1,3] Later, hematopoietic stem/progenitor cells or CD34+ cells from mobilized peripheral blood, bone marrow, or cord blood (CB) captured much attention because blood cells can be used immediately for reprogramming.[4–6] However, isolation of mobilized peripheral blood and bone marrow is invasive, time consuming and has potential risks for the donor, while harvesting CB cells has none of these limitations. In addition, >400,000 fully characterized and HLA-typed CB units are stored in public banks and are readily available for clinical therapy.[7] Moreover, CB has the youngest somatic cells and is expected to carry minimal genetic mutations induced by UV radiation.[8,9] Due to its unique advantages as donor cells for the production of clinical-grade human iPSCs, CB is believed to be one of the best sources for reprogramming. An additional advantage is the potential of converting CB banks into iPSC banks for allogeneic cell-based therapy.[10]

For clinical applications, transgene-free or footprint-free iPSCs need to be used to prevent potential adverse effects due to retroviral or lentiviral integration or due to the interference of residual expression of reprogramming factors on the differentiation of iPSCs into progenies of clinical interest.[11,12] Toward this goal, several approaches have been used for obtaining integration or transgene-free iPSCs, including the use of plasmids,[13] the Cre/loxP system,[14,15] adenoviruses,[16,17] piggyBac transposon,[18,19] minicircle DNA,[20] protein transduction,[21,22] Sendai virus,[23] and miRNA.[24] However, these methods suffer from low efficiency, require repetitive induction or selection, or require virus production. Synthetic modified mRNA might solve the problem,[25] but it requires the daily addition of mRNA by lipofection and CB CD34+ cells are among the most difficult to transfect by lipofection.

Several investigators have used the EBNA1-based episomal vector due to its unique features: (i) only one transfection of vector DNA by nucleofection is needed for efficient reprogramming, and (ii) the vector is lost in 5% or more cells after each cell division, leading to depletion of the episomal vector from cells after long-term passage. Recently, several groups have successfully used the pCEP4 episomal vector to generate footprint-free iPSCs.[26–28] However, in those studies, five to seven factors, including strong oncogenes like *MYC* and/or simian virus 40 large T antigen (*SV40LT*) were used, which raises safety concerns for the clinical use of iPSCs.

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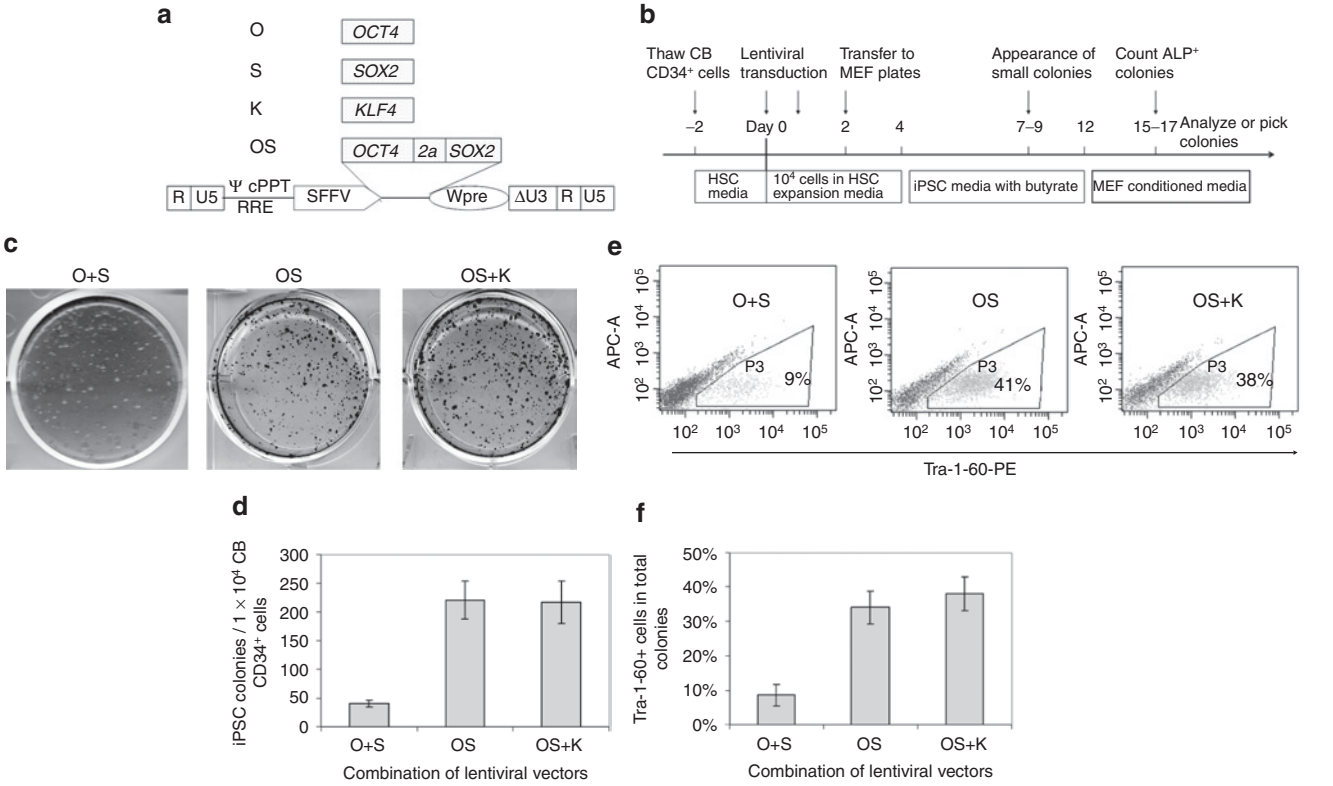

Figure 1 Lentiviral vector-mediated expression of OCT4 and SOX2 efficiently reprogram cord blood (CB) CD34$^+$ cells into induced pluripotent stem cells (iPSCs). (**a**) Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of the human reprogramming factor OCT4, SOX2, KLF4. Δ indicates the SIN design with partially deleted U3 of the 3′ long-terminal repeat. cPPT, central polypurine tract; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; Wpre, woodchuck post-transcriptional regulatory element; ψ, packaging signal; 2a, a self-cleavage site derived from equine rhinitis A virus. (**b**) Experimental strategy for reprogramming human CB CD34$^+$ cells using lentiviral vectors. (**c**) Representative alkaline phosphatase (ALP) staining of iPSC colonies 16 days after lentiviral transduction of 1 × 10^4 CB CD34$^+$ cells. O, OCT4; S, SOX2; K, KLF4. (**d**) Numbers of induced pluripotent stem cells (iPSCs) generated from 1 × 10^4 CB CD34$^+$ cells. $n = 3$. O+S vs. OS: $P < 0.05$. OS vs. OS+K: no significant difference. Data shown are presented as mean ± SEM. (**e**) Representative fluorescence-activated cell sorting (FACS) diagram of TRA-1-60 expression in cells undergoing reprogramming. Cells at day 16 after transduction were harvested and analyzed. (**f**) Percentages of TRA-1-60 positive cells in reprogramming cultures. O+S vs. OS: $P < 0.05$; OS vs. OS+K: no significant difference. Data shown are presented as mean ± s.e.m. ($n = 3$).

Earlier studies showed that OCT4 and SOX2 alone can reprogram CB cells into iPSCs, but at a very low efficiency.[9] We hypothesized that reprogramming efficiency might depend on expression levels of reprogramming factors, which largely relies on the promoters used. It is well known that the strength of promoters is contextual; several studies have shown that the spleen focus-forming virus (SFFV) promoter is stronger in primary hematopoietic cells or hematopoietic cell lines than many commonly used promoters like human elongation factor 1α (EF1α), cytomegalovirus, and A2UCOE (ubiquitous chromatin opening element).[29–32] Thus, we set out to determine whether iPSCs can be efficiently generated from CB CD34$^+$ cells with the SFFV promoter being used to drive expression of *OCT4* and *SOX2*.

RESULTS

Balanced expression of *OCT4* and *SOX2* by a lentiviral vector efficiently reprograms CB CD34$^+$ cells into iPSCs

It has been reported that overexpression of *OCT4* together with *SOX2* (O+S) using a retroviral vector in 2 individual constructs can reprogram CB CD133$^+$ cells into iPSCs.[9] However, the efficiency is as low as 0.002–0.005%, making this approach impractical for many applications. We hypothesized that the low efficiency might be due to low-level expression of the reprogramming factors O+S mediated by retroviral vectors. To test this assumption, we cloned reprogramming factors into a lentiviral vector driven by a strong promoter SFFV (**Figure 1a**).

As detailed in **Figure 1b** and the Materials and Methods section, CB CD34$^+$ cells were transduced with lentiviral vectors that express reprogramming factors followed by iPSC generation by culturing transduced cells on mouse embryonic fibroblasts (MEFs). Of interest, in the O+S condition, dozens of small colonies were observed in each well as early as 4–5 days after seeding transduced CB cells onto MEF layers, however, morphologically iPSC-like cells did not appear until a week later (data not shown). Analysis of these non-iPSCs by flow cytometry indicated that many cells expressed mesenchymal markers (data not shown). We also tested the combination of *OCT4* and *SOX2* (abbreviated as OS for clarity) in a single vector with the use of self-cleavage peptide sequence 2a. In this condition, no colonies were observed in the first week, and the first iPSC-like colonies appeared at 8–10 days after CB transduction. These data suggest that balanced expression of *OCT4* and *SOX2* may inhibit the outgrowth of non-iPSCs.

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In the O+S condition, we routinely observed 300–600 total colonies from 10,000 transduced CB CD34$^+$ cells 2 weeks after transduction. However, the majority of colonies were morphologically non-iPSCs and alkaline phosphatase (ALP) staining showed that ~20% of the colonies were iPSC-like (**Figure 1c**). In the OS condition, we observed 200–250 colonies in each well, with ~80% of the colonies being morphologically iPSCs, which was further confirmed by ALP staining (**Figure 1c,d**). In agreement with these results, fluorescence-activated cell sorting (FACS) analysis of the cells in the reprogramming cultures showed that only 9% of the cells in the O+S condition expressed the iPSC marker TRA-1-60, whereas ~40% of the cells in the OS condition were TRA-1-60 positive (**Figure 1e,f**).

Together, our findings demonstrate that OCT4 and SOX2 alone can efficiently reprogram CB cells into iPSCs and that balanced expression of the two factors that are linked with a 2a self-cleavage peptide sequence can increase reprogramming efficiency and inhibit growth of non-iPSC colonies.

KLF4 does not increase efficiency of lenti SFFV-OS-mediated reprogramming

Because the use of additional factors has been shown to boost reprogramming efficiency, we tested the effects of including other factors like KLF4 in reprogramming. In sharp contrast to expectations, we found that the addition of KLF4 (K) to OS did not increase the reprogramming efficiency. This surprising finding is unlikely to be explained by differential expression levels of reprogramming factors because the same OS vector was used in both conditions, and the expression of KLF4 was confirmed in preliminary studies. In OS conditions with and without K, 2% of transduced CB cells were successfully converted into iPSCs and ~40% of cells in the reprogramming culture expressed the iPSC marker TRA-1-60 (**Figure 1c–f**). This data suggests that the expression of OS, driven by the SFFV promoter, is sufficient to reprogram CB CD34$^+$ cells at high efficiency and addition of other factors like KLF4 does not significantly increase the reprogramming efficiency.

Efficiency of OS-mediated reprogramming depends on OS expression levels

Having observed up to a 1,000-fold higher efficiency in converting CB cells into iPSCs by OS compared to the previous report,[9] we speculated that differences in the expression levels of OS might explain the large difference in reprogramming efficiency. Transgene expression levels are largely determined by the strength of promoters; we thus cloned lentiviral vectors in which green fluorescent protein (GFP) expression is driven by the PGK, EF1, or the SFFV promoter to determine the strength of these promoters in CD34$^+$ cells (**Figure 2a**). FACS analysis showed that GFP expression driven by the PGK or the EF1 promoters is ~85% or ~60% lower than expression driven by the SFFV promoter in CB CD34$^+$ cells (**Figure 2b,c**). We reasoned that GFP is more stable than transcription factors; the GFP intensity may not reflect OCT4 or SOX2 expression levels. To address this issue, we cloned *OCT4GFP* fusion gene-expressing vectors driven by the three promoters. In this system, GFP is fused to the protein of interest. Thus the GFP expression, as measured by fluorescence intensity, can reflect the expression level of its fusion partner.[33] Similarly, we observed that the SFFV promoter drove highest level expression of OCT4GFP in CB CD34$^+$ cells, followed by the EF1 and the PGK promoters (**Figure 2d**). Of note, GFP intensity was decreased by ~20-fold in OCT4GFP-transduced cells, as compared to GFP-transduced cells, and the differences in expression of OCT4GFP were less pronounced than that of GFP, which reflect the rapid turnover of OCT4 in CB CD34$^+$ cells. Together, these data suggest that the SFFV promoter drives significantly higher levels of transgene expression in CB CD34$^+$ cells than the PGK or EF1 promoters.

To investigate the effects of low OS expression on reprogramming efficiency, we used the weaker PGK and EF1 promoters to drive OS expression. In more than five independent experiments, no iPSC colonies could be generated from 1×10^4 CB CD34$^+$ cells that were transduced with lenti PGK-OS or lenti EF1-OS vectors (**Figure 2e**). Given that expression of OCT4 is decreased by ~50% when driven by EF1 as compared to the SFFV promoter (**Figure 2d**), this observation suggests that a 50% decrease in OS expression could lead to reprogramming failure. In hopes of increasing OS expression and thereby reprogramming efficiency, we synthesized an OS gene (*synOS*) that was codon optimized by DNA 2.0 (Menlo Park, CA). In contrast to our expectation, expression of OS at the protein level by synOS was ~20% lower than the wild-type human OS. Of note, this small decrease in OS expression translated into a fourfold decrease in reprogramming efficiency (data not shown). This observation further supports our conclusion that OS-mediated high-efficiency reprogramming critically depends on OS expression levels, and a slight decrease in OS expression leads to a substantial drop in reprogramming efficiency, whereas a 50% decrease results in reprogramming failure.

MYC and KLF4 facilitate reprogramming when OS expression levels are low

Having found that low-level OS expression is insufficient to induce CB reprogramming, we further asked whether this can be rescued by MYC and KLF4. As anticipated, in CB CD34$^+$ cells that were transduced with EF1-OS or SFFV-MK alone, no iPSCs were generated. In contrast, after transduction of CB CD34$^+$ cells with both EF1-OS and SFFV-MK, 0.1% cells were converted into iPSCs (**Figure 2f**). ALP staining and FACS analysis of iPSCs did not show any obvious differences in the expression of pluripotency markers when compared with iPSCs generated with SFFV-OS (data not shown). Of interest, when *MYC* and *KLF4* expression was driven by the EF1 promoter, which leads to lower expression levels, no iPSCs could be generated (data not shown). Together, these findings suggest that high-level expression of OS alone is sufficient for CB reprogramming, whereas reprogramming under low-level OS expression requires other reprogramming factors.

Generation of footprint-free iPSCs using an episomal vector

The successful generation of iPSCs with a lentiviral vector that expresses *OCT4* and *SOX2* alone prompted us to ask whether this approach would also work in a nonviral system. To test this, we shuttle cloned SFFV-OS from the lentiviral vector construct

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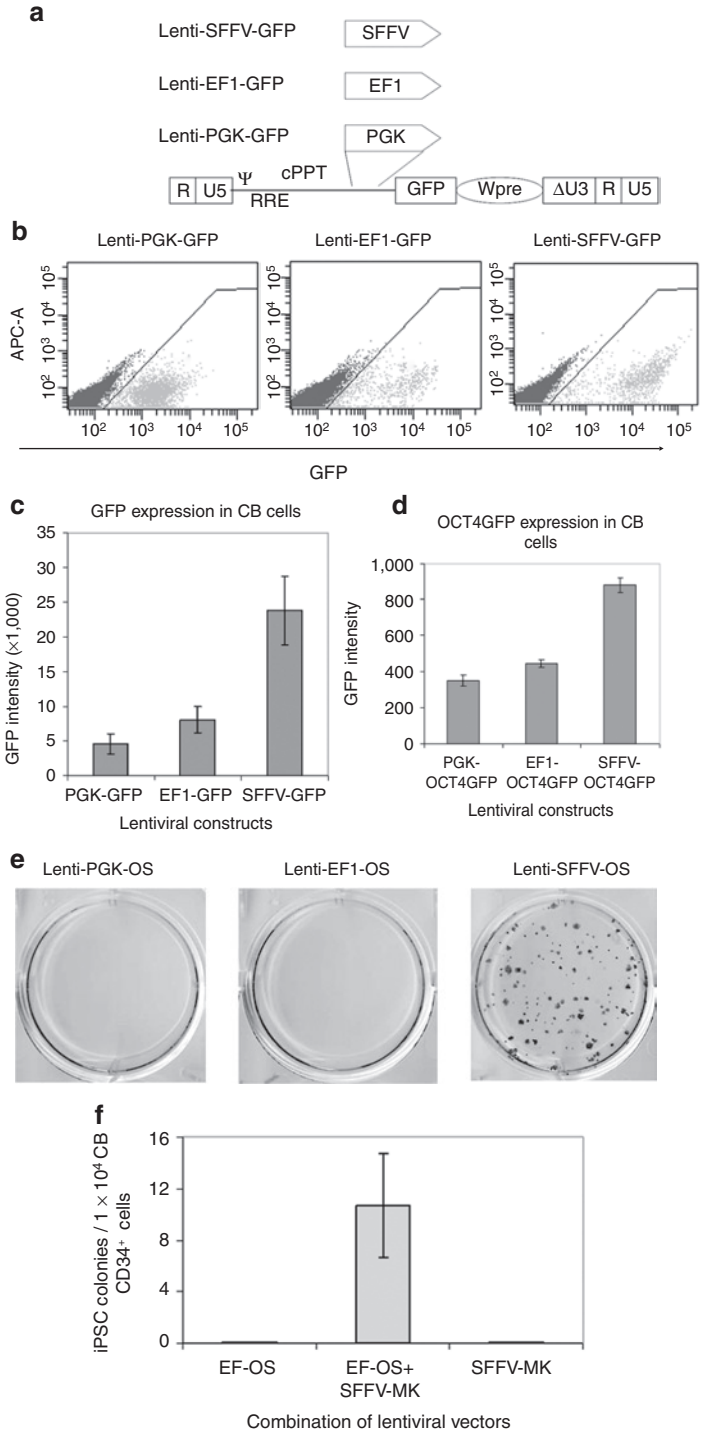

Figure 2 Efficiency of OCT4 and SOX2-mediated reprogramming depends on gene expression levels. (**a**) Schematic of the self-inactivating (SIN) lentiviral vector backbones for expression of green fluorescent protein (GFP). Δ indicates the SIN design with partially deleted U3 of the 3′ long-terminal repeat. cPPT, central polypurine tract; EF1, elongation factor-1α promoter; PGK, phosphoglycerokinase promoter; RRE, rev-responsive element; SFFV, spleen focus-forming virus U3 promoter; Wpre, post-transcriptional regulatory element; ψ, packaging signal. (**b**) Representative levels of GFP expression driven by three different promoters in cord blood (CB) $CD34^+$ cells. Fluorescence-activated cell sorting (FACS) analysis was conducted at 3 days post-transduction. (**c**) Distinct GFP expression levels driven by three different promoters in CB $CD34^+$ cells. $n = 3$. PGK-GFP vs. EF-GFP: $P = 0.05$; EF-GFP vs. SFFV-GFP: $P < 0.05$. (**d**) Increased expression of OCT4GFP fusion gene driven by SFFV promoter compared to PGK and EF1 in CB $CD34^+$ cells. FACS analysis was conducted at 3 days post-transduction. $n = 3$. PGK-OCT4GFP vs. EF1-OCT4GFP: $P = 0.06$; EF1-OCT4GFP vs. SFFV-OCT4GFP: $P < 0.01$. (**e**) Alkaline phosphatase (ALP) staining for iPSC cultures from CB cells transduced with PGK-OS, EF1-OS, and SFFV-OS. Note that no colonies were generated in PGK-OS, EF1-OS conditions. (**f**) Expression of MYC and KLF4 rescues failure of low level OS expression driven by EF1 promoter in generating induced pluripotent stem cells (iPSCs) from CB $CD34^+$ cells. Graphed data are presented as mean ± SEM ($n = 3$).

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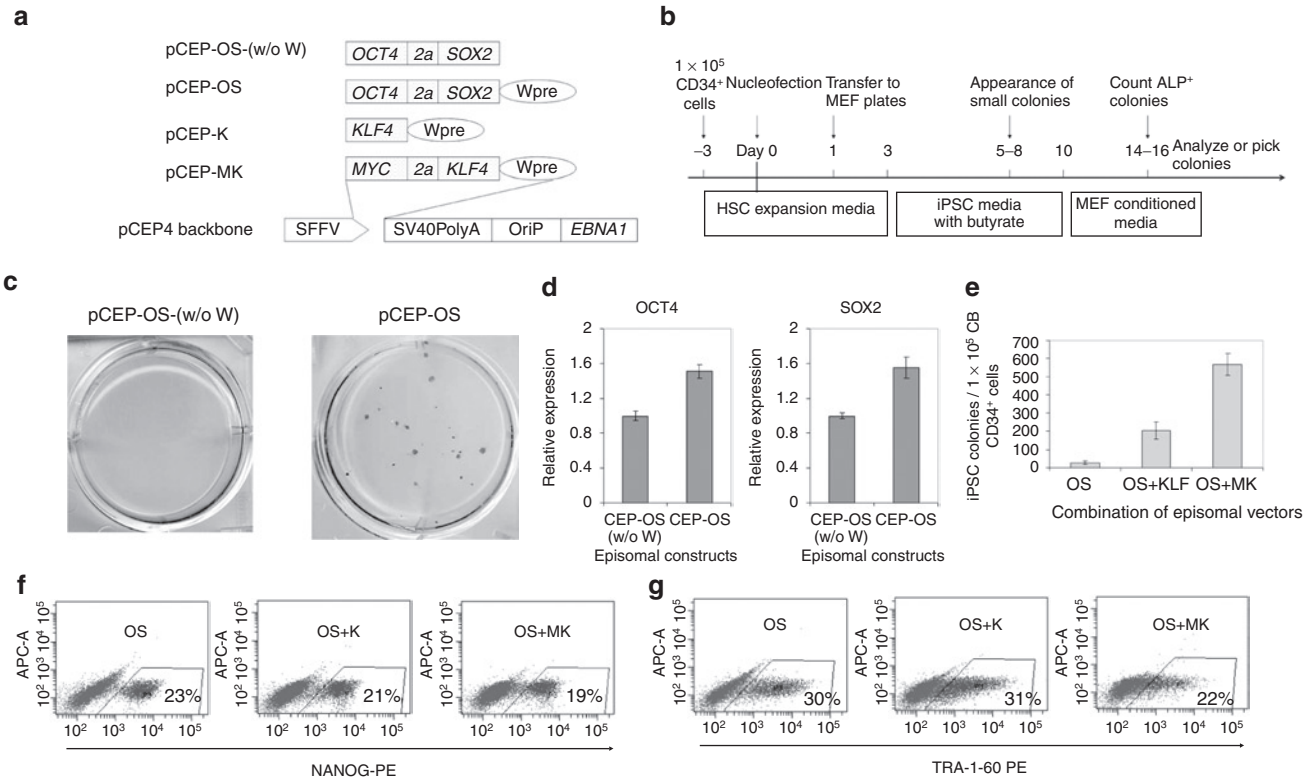

Figure 3 OCT4 and SOX2-mediated reprogramming using episomal vectors. (**a**) Schematic of episomal vectors used in this study for conversion of cord blood (CB) CD34$^+$ cells into induced pluripotent stem cells (iPSCs). Reprogramming factors were cloned into the pCEP4 backbone; their expression is driven by spleen focus-forming virus U3 promoter (SFFV). 2a is a self-cleavage site derived from equine rhinitis A virus. Wpre, post-transcriptional regulatory element; SV40PolyA, polyadenylation signal from SV40 virus; OriP, EBV origin of replication; EBNA1, Epstein–Barr nuclear antigen 1, which plays essential roles in replication and persistence of episomal plasmid in infected cells. (**b**) Experimental strategy for reprogramming human CB CD34$^+$ cells using EBNA1-based episomal vectors. (**c**) Representative alkaline phosphatase (ALP) staining shows that inclusion of the Wpre element in the episomal vector pCEP-OS (w/o W) results in successful reprogramming. $n = 3$. Colonies are from 1 × 10^5 CB CD34$^+$ cells. (**d**) Inclusion of Wpre element in the CEP episomal vector increases gene expression. 293T cells were infected with same amount of plasmids. 3 days after transfection, OCT4 and SOX2 expression was examined by intracellular staining and fluorescence-activated cell sorting (FACS) analysis. $n = 3$. pCEP-OS (w/o W) vs. pCEP-OS: $P < 0.05$. (**e**) Numbers of ALP positive iPSC colonies at 16 days post-transfection of 1 × 10^5 CB CD34$^+$ cells with pCEP-OS (OS) and pCEP-K (K) or pCEP-MK (MK). $n = 3$. OS vs. OS+K: $P < 0.05$; OS+K vs. OS+MK: $P < 0.05$. Expression of the iPSC markers (**f**) NANOG and (**g**) TRA-1-60 in cultures reprogrammed using three different combinations of episomal vectors. Cells were harvested for FACS analysis 20 days after nucleofection.

into a pCEP4 EBNA1/OriP-based episomal vector (**Figure 3a**). To generate iPSCs, 1 × 10^5 CB CD34$^+$ cells were cultured in Iscove's modified Dulbecco's medium/10% fetal bovine serum with cytokines SCF, FL, and TPO. After 3 days of culture, the total cell number increased by approximately fivefold and all the cells were harvested for nucleofection with the pCEP-OS (w/o W) plasmid (**Figure 3b**). In three independent experiments, we failed to generate any iPSCs (left panel of **Figure 3c**). We reasoned that this failure might be due to the low-level expression of OS mediated by this vector. We then cloned woodchuck post-transcriptional regulatory element (Wpre), a post-transcriptional regulatory element that is commonly used in lentiviral systems to enhance gene expression levels, into the pCEP-OS (w/o W) plasmid (**Figure 3a**). As expected, the inclusion of Wpre in the episomal vector led to a 50% increase in OCT4 expression and a 55% increase in SOX2 expression (**Figure 3d**). Using pCEP-OS, we successfully generated ~20 iPSC colonies from the progeny of 1 × 10^5 freshly thawed CB CD34$^+$ cells (**Figure 3c,e**).

To better compare our improved vector with published results, we evaluated the effects of KLF4 or MK (MYC and KLF4) together with OS on the efficiency of CB reprogramming. With the addition of KLF4, the reprogramming efficiency increased by eightfold, and further inclusion of MYC led to an additional threefold increase (**Figure 3e**). Of interest, the appearance of the first iPSC-like colonies was observed at 9–10, 6–7, and 4–5 days after cells were transfected with episomal OS, OS+K, and OS+MK plasmids, respectively. This data suggests that addition of KLF4 and/or MYC accelerates the reprogramming process. Of note, using two episomal vectors that express four factors, we generated up to 600 iPSC colonies from 1 × 10^5 CB CD34$^+$ cells, compared to 80 colonies from the same amount of CB CD34$^+$ cells even with five factors (OSMK + LIN28).[26] These data suggest that our improved episomal vector is substantially more efficient in reprogramming CB cells into iPSCs than previously reported.

We conducted further tests to examine the differences in the expression of pluripotency markers between iPSCs generated with the three different combinations of episomal vectors. Immunostaining and FACS analysis showed that 20–30% of cells expressed the iPSC markers NANOG and TRA-1-60 in all the

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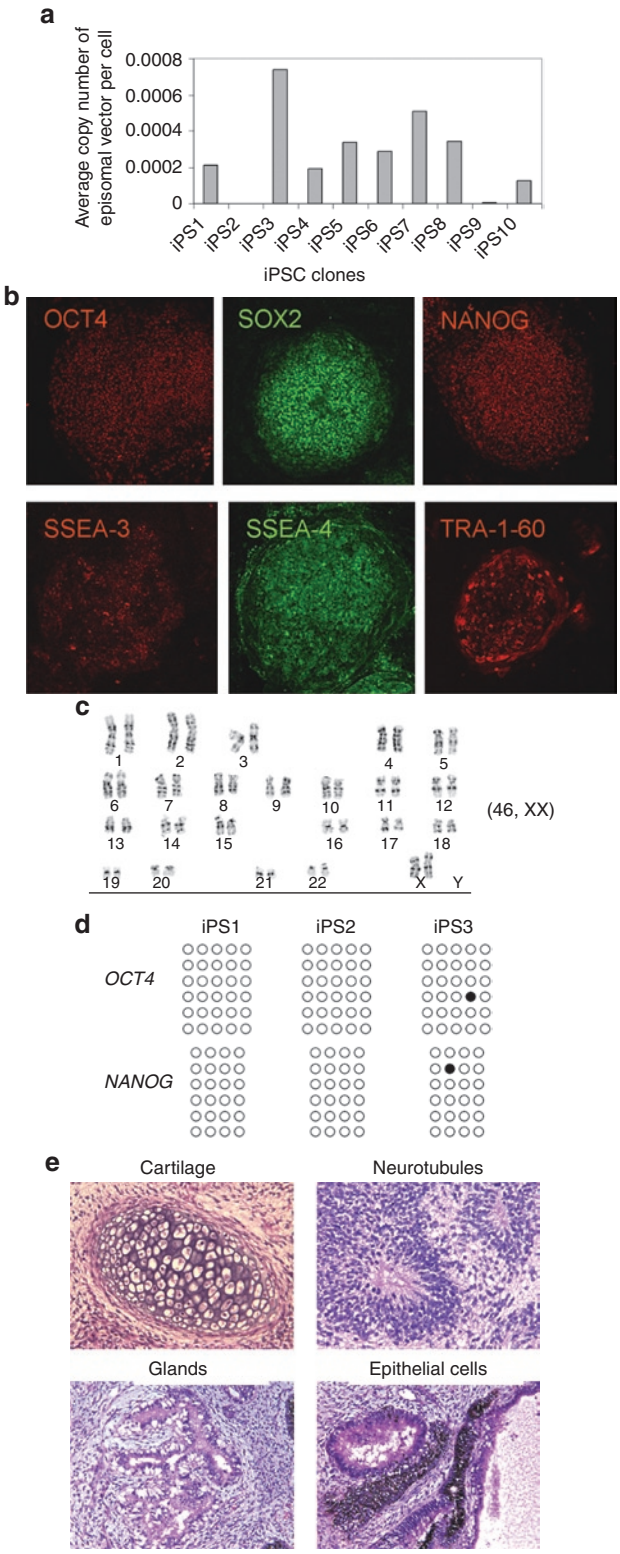
three combinations, whereas including MYC appeared to decrease the portion of Tra-1-60 positive iPSCs in reprogramming culture (Figure 3f,g).

Taken together, these data demonstrate that we have developed an episomal vector in which increased expression of reprogramming factors leads to efficient reprogramming of CB cells into iPSCs. We show for the first time, that iPSCs can be generated with the episomal vector that expresses only OCT4 and SOX2.



Characterization of iPSC colonies generated with the pCEP-OS plasmid

To characterize iPSCs, we randomly picked 10 colonies from the pCEP-OS reprogrammed cultures and passaged iPSCs for >3 months. Real-time PCR analysis with two pairs of primers showed that at passage 0, ~0.5 copy of the pCEP-OS plasmid per cell could be detected. After eight passages, the average copy number of residual CEP plasmid decreased to 0.001–0.007/genome and in 2 out of 10 clones, the presence of CEP plasmid was undetectable (Figure 4a). After 12 passages, residual episomal plasmid was disappeared in the majority of clones (data not shown). This finding is consistent with previous reports showing that the presence of episomal vector is undetectable in most iPSC colonies after 10–14 passages.[26]

To extensively characterize pCEP-OS generated iPSCs, we selected several clones for a series of tests. Immunostaining of iPSC colonies showed that they expressed typical human iPSC-specific transcription factors OCT4, SOX2, NANOG, and surface markers SSEA-3, SSEA-4, and Tra-1-60 (Figure 4b). Karyotype analysis indicated a normal human karyotype for all the clones tested; one representative is shown in Figure 4c. Sulphite sequencing showed that both the *OCT4* and *NANOG* promoters were demethylated in three randomly picked iPSC clones (Figure 4d). When injected into immunodeficient NSG mice, iPSCs formed teratomas consisting of derivatives of all three embryonic germ layers, demonstrating the pluripotency of these iPSCs (Figure 4e). Together, these data suggest that *bona fide* transgene-free iPSCs can be generated from human CB CD34$^+$ cells by nucleofection of a pCEP episomal plasmid that expresses OCT4 and SOX2 alone.

DISCUSSION

Here, we report that iPSCs can be generated from human CB CD34$^+$ cells in 2–3 weeks with the use of OCT4 and SOX2 alone. We found that lentiviral vector-mediated transduction of OS is sufficient to reprogram 2% of transduced CB CD34$^+$ cells into iPSCs. This efficiency is up to 1,000-fold higher than previously reported,[9] which is attributed to the SFFV promoter-mediated high-level expression of OS. Furthermore, with the use of an

Figure 4 Characterization of induced pluripotent stem cells (iPSCs) generated with pCEP-OS. (**a**) Copies of residual episomal vectors after eight passages as indicated by real-time PCR. Data shown are from one pair of primers. Similar results were obtained with second pair of primers. (**b**) Immunohistochemistry analysis of a representative iPSC line showing expression of indicated pluripotency markers. Images were captured using the Zeiss LSM 710 confocal microscope with a ×10 objective. (**c**) A representative karyogram of an iPSC clone. All analyzed iPSC clones showed a normal karyotype. (**d**) Bisulphite genomic sequencing of the *OCT4* and *NANOG* promoters indicates demethylation in three independent clones. Each horizontal row of circles represents an individual sequencing reaction of a given amplicon. Open and filled circles represent unmethylated and methylated CpG dinucleotides, respectively. (**e**) Hematoxylin and eosin (H&E) staining of representative teratoma from pCEP-OS cord blood (CB) iPSCs shows derivatives of three embryonic germ layers. Cartilage (mesoderm); neurotubules with rosettes (ectoderm); glands (endoderm); retina epithelial cells with pigments (ectoderm). Images were acquired using the Olympus microscope with a ×20 objective.

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improved OS-expressing episomal vector in which the inclusion of Wpre increases transgene expression by 50%, 20 footprint-free iPSCs can be generated from 1×10^5 CB $CD34^+$ cells, an amount that can be purified from ~1 ml of CB. To the best of our knowledge, this is the first report that footprint-free iPSCs can be generated with only two factors.

Striking progress in iPSC reprogramming has been made over the past several years. iPSCs can be generated from almost any kind of mammalian cells. However, recent reports that describe exceedingly high rates of genetic point mutations and gene copy number variations have shifted the research focus from reprogramming efficiency to reprogramming safety.[34,35] Two parameters are likely to be the key to the generation of safe iPSCs for clinical use: cell source and reprogramming method. It is widely accepted that CB is one of the best cell sources for reprogramming. However, one of the four transcription factors originally used by Yamanaka and Takahashi for cell reprogramming, MYC, is oncogenic. Overexpression of *MYC* has been shown to induce malignant transformation,[36] Another commonly used reprogramming booster *SV40LT* is also oncogenic. SV40LT functions by inhibition of the p53 and Rb-family of tumor suppressors and ectopic expression of SV40LT induces in vitro cellular transformation and *in vivo* tumorigenesis.[37] Although expression of reprogramming factors is only required for ~2 weeks, this short-term exposure to MYC may elicit adverse effects on genomic stability.[38] Therefore, we propose that an ideal combination of reprogramming factors should be devoid of factors whose overexpression has been demonstrated to induce cellular transformation and in vivo tumorigenesis.

With safety considerations in mind, we initiated experiments to optimize reprogramming conditions using only OS expressed by a lentiviral vector. We found that high-level expression of OS, driven by a strong promoter SFFV, led to the conversion of 2% of transduced cells into iPSCs. This efficiency is up to 1,000-fold higher than previously reported for these factors.[9] An ~20% decrease in OS expression levels led to a fourfold decrease in efficiency. Moreover, when OS expression was decreased by 50% or more with the use of promoters like EF1 and PGK, no iPSCs could be generated from CB $CD34^+$ cells. These findings establish that reprogramming of CB cells with OS critically depends on the expression levels of these genes. It is tempting to speculate that high-level expression of OCT4 and SOX2 alone could also reprogram other cells like fibroblasts. However, SFFV is not necessarily a strong promoter in cell types other than hematopoietic cells. For instance, the EF1 promoter drives higher-level expression of transgenes in fibroblasts than the SFFV promoter (data not shown).

To generate footprint-free iPSCs, we used an episomal vector. In the absence of the Wpre element, the OS-expressing pCEP episomal vector was insufficient to reprogram CB cells into iPSCs. However, an improved episomal vector design that included Wpre at the 3′ end of the transgene and in front of the PolyA signal, led to the successful generation of iPSCs. Of note, sodium butyrate was used for ~10 days in our reprogramming culture. Omitting sodium butyrate led to a considerable decrease in reprogramming efficiency (data not shown). This data suggests that sodium butyrate is also crucial for episomal vector-mediated cellular reprogramming. Characterization of iPSC colonies showed no differences in iPSC quality between different combinations of reprogramming factors, as evidenced by a series of *in vitro* and *in vivo* tests. Moreover, after 12 passages, no integration or residual episomal plasmid can be identified in most clones by sensitive real-time PCR analysis. However, a caveat is that this does not necessarily mean there is no integration of small fragments in these iPSC clones. Such fragments can only be detected by whole genome sequencing. While the reprogramming efficiency mediated by pCEP-OS is relatively low, this system is capable of generating sufficient numbers (20 iPSCs/ml of CB) of iPSCs for allogeneic cell therapy.

The generation of transgene-free iPSCs from CB cells has recently been reported by several groups. Yu and colleagues found that the use of episomal vectors expressing seven factors can highly efficiently reprogram CB cells; however no iPSCs could be generated in the absence of *SV40LT* expression.[27] Using a 5-in-1 vector (OSMK and *LIN28*), Cheng and colleagues were able to generate 80 iPSCs from 1×10^5 CB $CD34^+$ cells.[26] From the same amount of cells, we can generate ~20 iPSCs with OS alone, and up to 600 iPSCs with OSMK. Considering that the addition of LIN28 increases reprogramming efficiency by three to fivefold,[39] our improved vector is at least 20-fold more efficient in reprogramming CB cells than plasmids used in previous studies. Our success is attributed to the inclusion of two features in the vector design: (i) the SFFV promoter, which drives higher levels of transgene expression in hematopoietic cells than PGK, EF1 or other promoters; and (ii) the Wpre element, which increases transgene expression by 50%. Wpre is commonly used in lentiviral vectors to improve transgene expression;[30] our findings suggest that Wpre is also functional in episomal plasmids and possibly other DNA vectors such as adenoviral vectors.

In summary, we are the first to report the successful generation of transgene-free human iPSCs with the use of OCT4 and SOX2 alone. All OS-reprogrammed iPSCs examined in our studies showed normal karyotypes. Future studies that compare genetic instability and mutation rates in iPSCs generated with OS alone versus combinations that include oncogenic factors like MYC will be an important next step on the path to clinical application of iPSCs.

MATERIALS AND METHODS

Cord blood. The use of CB was approved by the institutional review board of Loma Linda University (LLU) and written informed consent was obtained from all participants. $CD34^+$ cells were purified with a $CD34^+$ Microbead Kit (Miltenyi Biotec, Auburn, CA).

Construction of lentiviral and episomal vectors. Human *OCT4*, *SOX2*, *MYC*, and *KLF4* cDNAs were purchased from Open Biosystems, Huntsville, AL and cloned into the pRRLSin.cPPT.PGK-GFP.WPRE lentiviral vector that was kindly provided by Luigi Naldini via Addgene, Cambridge, MA (Plasmid 12252).[40] Open reading frames of these reprogramming factors and PGK, EF1, or SFFV promoters were inserted into this vector by PCR cloning. For cloning OS or MK vectors, a 2A sequence was used to link *OCT4* and *SOX2*, or *MYC* and *KLF4*.[41] The EBNA1/OriP-based pCEP4 episomal vector was purchased from Invitrogen (Carlsbad, CA). For cloning pCEP-OS (w/o W), pCEP-OS, pCEP-K, or pCEP-MK vectors, the hygromycin resistance gene element and cytomegalovirus promoter were removed from the pCEP4 vector by digestion with endonucleases *Nru*I and

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*BamH*I, and inserts were cut from the counterparts of lentiviral vectors. All the constructs were verified by sequencing. For lentivirus production, a standard calcium phosphate precipitation protocol was used. Titers of 5–10 × 10^7/ml were routinely achieved in our lab after a 100-fold concentration by centrifugation at 6,000*g* for 24 hours at 4 °C.[42,43]

Generation of iPSCs using lentiviral vector. Thawed CB CD34$^+$ cells were cultured in hematopoietic stem cell culture condition: Iscove's modified Dulbecco's medium/10% fetal bovine serum supplemented with TPO, SCF, FL, and G-CSF each at 100 ng/ml, and IL-3 at 10 ng/ml.[44] Cytokines were purchased from ProSpec (East Brunswick, NJ). After 2 days prestimulation, 1 × 10^4 cells/well were seeded into non-TC treated 24-well plates that were precoated with RetroNectin (CH-296; Takara Bio, Shiga, Japan) for lentiviral transduction for 4–5 hours. A second transduction was conducted 24 hours later. One day after transduction, cells were harvested and transferred to 6-well plates, which were preseeded with a mitomycin C-inactivated CF-1 MEF feeder layer (Applied Stemcell, Menlo Park, CA). Passage five MEFs were used in our experiments. Cells were maintained in the hematopoietic stem cell culture condition for 2 more days before being replaced with iPSC media. The iPSC media used in our study is composed of knockout DMEM/F12 medium (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1 mmol/l GlutaMAX (Invitrogen), 2 mmol/l nonessential amino acids (Invitrogen), 1× penicillin/streptomycin (Invitrogen), 0.1 mmol/l β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 20 ng/ml FGF2 (ProSpec). To increase reprogramming efficiency, sodium butyrate[45] was added at 0.25 mmol/l from day 2–12, and cells were cultured under hypoxia[46] by placing culture plates in a Hypoxia Chamber (Stemcell Technologies, Vancouver, British Columbia, Canada) that was flushed with mixed air composed of 92%N_2/3%O_2/5%CO_2. Starting from day 10, MEF-conditioned medium was used. At day 14–16, ALP staining was conducted to quantitate iPSC colonies. Alternatively, all the colonies were harvested by Accutase (Innovative Cell Technologies, San Diego, CA) treatment for FACS analysis.

Immunostaining and flow cytometry. Staining for ALP was carried out using an ALP-staining kit (Stemgent, San Diego, CA) to quantitate iPSC colonies. For intracellular staining, cells were fixed for 30 minutes at room temperature in fixation buffer and permeabilization buffer (eBiosciences, San Diego, CA). After washing, cells were stained at room temperature for 2 hours with NANOG-PE (BD Pharmingen, San Diego, CA), followed by washing twice with permeabilization buffer. For staining of cell surface marker TRA-1-60-PE (Stemgent), cells were incubated with the antibody for 30 minutes at room temperature. Flow cytometric analysis was performed using FACS Aria II (BD Biosciences, San Jose, CA) with a 488-nm laser. Thirty thousand events were collected for each sample.

Episomal vector and nucleofection. Fresh or thawed 1 × 10^5 CB CD34$^+$ cells were cultured in Iscove's modified Dulbecco's medium/10% fetal bovine serum supplemented with TPO, SCF, and FL at 100 ng/ml. Three days later, cells were harvested for nucleofection with a total of 12 μg CEP plasmid DNAs. Human CD34 Cell Nucleofector Kit (Lonza, Walkersville, MD) was used. Nucleofection was performed with Amaxa Nucleofector II using program U-008. Immediately after nucleofection, cells were cultured in a CH-296 pretreated well plate to facilitate the CB cell recovery. The next day, half of the cells were transferred to each well of MEF-coated 6-well plates. Cells were cultured the same way as for reprogramming with lentiviral vector. The total number of iPSC colonies was counted on day 16 post-transfection after ALP staining. At day 14–17, colonies were picked for further culture or harvested for FACS analysis.

Confocal imaging. For immunostaining of iPSC colonies, iPSCs were cultured in chamber slides for 4–5 days. Cells were treated with fixation buffer and permeabilization buffer (eBiosciences) for 30 minutes before being stained overnight with PE or FITC-conjugated antibodies OCT4 (eBiosciences), SOX2 (BD Pharmingen), NANOG (BD Pharmingen), SSEA-3 (eBiosciences), SSEA-4 (eBiosciences), and TRA-1-60 (Stemgent). The samples were washed twice with permeabilization buffer, counterstained with 4′,6-diamidino-2-phenylindole and coverslipped before being imaged. Imaging was performed using the Zeiss LSM 710 NLO laser scanning confocal microscope with a ×10 objective at the LLU Advanced Imaging and Microscopy Core. High resolution monochrome image was captured using a Zeiss HRm CCD camera (Thornwood, NY).

Teratoma assay. The use of NOD/SCID/IL2RG$^{-/-}$ (NSG) immunodeficient mice for the teratoma formation assay was approved by the Institutional Animal Care and Use Committee at LLU. NSG mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the LLU animal facility. Approximately 1 × 10^6 iPSCs were harvested by Dispase (Invitrogen) digestion, washed with culture medium and resuspended in 200 μl DMEM/F12 diluted (1:1) Matrigel solution (BD, San Jose, CA). Cells were injected into the subcutaneous tissue above the rear haunch of NSG mice. At 6–8 weeks after injection of iPSCs, teratomas were dissected and fixed in 10% formalin. After sectioning, samples were embedded in paraffin and stained with hematoxylin and eosin and analyzed by a board certified pathologist.

Bisulphite sequencing. Bisulphite sequencing of genomic DNA from iPSC clones was used to assess methylation status of OCT4 and NANOG promoter. Genomic DNA was purified from human iPSCs by DNeasy Kit (Qiagen, Valencia, CA). The conversion of unmethylated cytosines to uracil was carried out using EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA). Approximately 1 μg genomic DNA was treated in each reaction, and 4 μl of elution was used for each PCR. PCR with primers OCT4-mF3/R3 and NANOG-mF3/R3, which were used by other investigators,[47] was carried out using Titanium Taq polymerase (Clontech Laboratories, Mountain View, CA): The cycling conditions were 95°C 10 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and finally 72 °C for 7 minutes. The PCR products were cloned into a pJET1.2 vector (Fermentas, Glen Burnie, MD) and sequenced by MCLAB (San Francisco, CA).

Karyotyping and G-banding. GTG-banding chromosome analysis was carried out in the LLU Radiation Research Laboratories. Standard DNA spectral karyotyping procedures were followed and a HiSKY Complete Cytogenetic System was used (Applied Spectral Imaging, Vista, CA). For each clone, 10 metaphases were analyzed and karyotyped. The data were interpreted by a certified cytogenetic technologist.

Real-time PCR. To determine the average copy numbers of residual or integrated CEP vector in iPSC clones, real-time PCR analysis was performed. Total DNA (genomic and episomal) was extracted from iPSCs using the DNeasy kit from Qiagen. Equal amounts of DNA (100 ng) isolated from naive cells (before nucleofection) were used as negative control, while a manual mixture of 1 copy pCEP-OS vector per genome was used as a positive control to calculate the average copy numbers of residual episomal vector in each iPSC after multiple passages. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on 7500 Fast Real-Time PCR System (Applied Biosystems). Two sets of primers were used to detect CEP plasmid DNA (in either episomal or integrated form): EBNA1-F: 5′-TTTAATACGATTGAGGGCGTCT-3′, EBNA1-R: 5′-GGTTTTGAAGGATGCGATTAAG-3′; OSW-F: 5′- GGATTACAAGG ATGACGACGA-3′, OSW-R: 5′- AAGCCATACGGGAAGCAATA-3′. The amplification program consisted of 50 °C for 2 minutes and 95 °C for 10 minutes, and was followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Statistical analysis. Data are presented as mean ± s.e. of the mean (s.e.m.). Two-tailed Student *t*-test was performed. *P* value of <0.05 was considered statistically significant.

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